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13. ABSTRACT (Maximum 200 Words)

hSpry2 has been identified as a modulator of RTK signaling pathways, and growth factor stimulation regulates hSpry2 expression and function establishing complex regulatory network. We are interested in identifying factors that control the biological activity of hSpry2. Previously our lab has shown that growth factor stimulation induces the proteasomal degradation of hSpry2 through poly-ubiquitination by E3 ubiquitin ligase, c-Cbl. Recently we found that hSpry2 is also mono-ubiquitinated. In order to characterize the function of hSpry2 mono-ubiquitination, we generated an hSpry2 ubiquitination-deficient mutant by site directed mutagenesis. Indirect immunofluorescent microscopy showed that hSpry2 ubiquitination-deficient mutant localizes to Golgi apparatus whereas wild-type hSpry2 localizes to both the Golgi and the cytoplasmic vesicular structures. Cytoplasmic vesicular localization of the mutant was regained by fusing a single ubiqutin molecule to it, strongly suggesting the involvement of mono-ubiquitination in intracellular trafficking. Taken together, these results indicate that hSpry2 monoubiquitination serves as a signal for its Golgi to endosome trafficking.

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Introduction

Growth factor receptor tyrosine kinases (RTKs) control a wide range of cellular events including proliferation, differentiation, migration and survival. In normal cells, RTK signaling is tightly regulated to ensure appropriate biological outcome. This control is achieved through the coordinated action of negative regulators. Identifying the mechanisms by which the functions of these negative regulators are controlled will give us a better understanding of the complexity of RTK signaling networks. hSpry2 is a recently identified inhibitor of RTK signaling [1-3]. Results generated prior to the receipt of funding indicated that hSpry2 is modified by a ubiquitin chain (poly-ubiquitination) [4,5] and a single ubiquitin molecule (mono-ubiquitination) that are important for the proteasomal degradation and protein trafficking, respectively. We have investigated the functional significance of hSpry2 mono-ubiquitination during the current funding period. As discussed below, progress has been made in generating an hSpry2 ubiquitination-deficient mutant, and preliminary results have pointed to a possible role for mono-ubiquitination in the intracellular trafficking of hSpry2.

Body

Initial work focused on generating an hSpry2 mono-ubiquitination-deficient mutant construct. Since lysine residues on target proteins are modified by ubiquitin molecules, site directed mutagenesis using polymerase chain reaction (PCR) was performed to mutate possible target lysine(s). Mutant DNAs were ligated to a pCGN vector that contains an HA tag at the N-terminus to detect the transfected species with an anti-HA antibody. Mutation of a single lysine residue or a combination of mutations of several lysine residues failed to abolish hSpry2 mono-ubiquitination. This suggests that an alternative lysine residue can be ubiquitinated when a proper target is missing. To circumvent this problem, an hSpry2 ubiquitination-deficient mutant in which all lysine residues were mutated to arginine was generated and designated as an hSpry2 lysine (K) Null Mutant (hSpry2 KNM). The failure of this mutant to undergo mono-ubiquitination was established by ubiquitination assays (Figure 1).

To investigate a possible role of hSpry2 mono-ubiquitination in protein trafficking, hSpry2 wild type and KNM localization were examined by indirect immunofluorescent microscopy using an anti-HA antibody. The wild-type hSpry2 was localized to the perinuclear region and cytoplasmic vesicular structures of the endosomal compartment, hSpry2 KNM, in contrast, showed only perinuclear region staining (Figure 2). Further characterization of the identity of perinuclear region staining indicated that it corresponds to the trans-Golgi apparatus (Data not shown).

In order to investigate the involvement of hSpry2 mono-ubiquitination in targeting the protein to the cytoplasmic vesicular structures, the ubiquitin-deficient hSpry2 construct was fused N-terminally to ubiquitin (Ub/SpKNM) (Figure 3A). To prevent poly-ubiquitin chains from being generated using lysine residues on ubiquitin, three lysine residues on ubiquitin (K29, K48 and K68) that can be conjugated by another ubiquitin were mutated to arginine residues. To block the cleavage of ubiquitin molecules from hSpry2 KNM protein by a deubiquitinating enzyme, the last two glysine residues that serve for cleavage sites on ubiquitin were deleted. The advantage of this construct is

that it mimics constitutively mono-ubiquitinated hSpry2 species. Indirect immunofluorescent microscopy using either anti-HA or anti-Spry antibody showed that the phenotype of hSpry2 KNM was fully rescued by a fusion of single ubiquitin moety (Ub/SpKNM) (Figure 3B). Data obtained from these experiments strongly suggest that hSpry2 mono-ubiquitination targets protein from Golgi to cytoplasmic vesicles.

Studies concerning the biological significance of the Golgi localization of hSpry2 are currently in progress. It has been demonstrated that a subpopulation of H-Ras is located and activated on the Golgi membrane by a guanine nucleotide exchange factor, RasGRP [6,7]. This signaling is therefore quite distinct from the more classical activation of Ras at the plasma membrane that is mediated by Sos, and is suggested to be responsible for the prolonged activation of ERK/MAPK [8]. Since Spry is an antagonist of RTK signaling, it is possible that Golgi localized hSpry2 may affects H-Ras activation in the Golgi. In order to test this idea, we will employ imaging techniques designed to monitor the activation of Ras in the Golgi. In addition, we will perform studies aimed at determining the significance of Spry localized to endosomal structures.

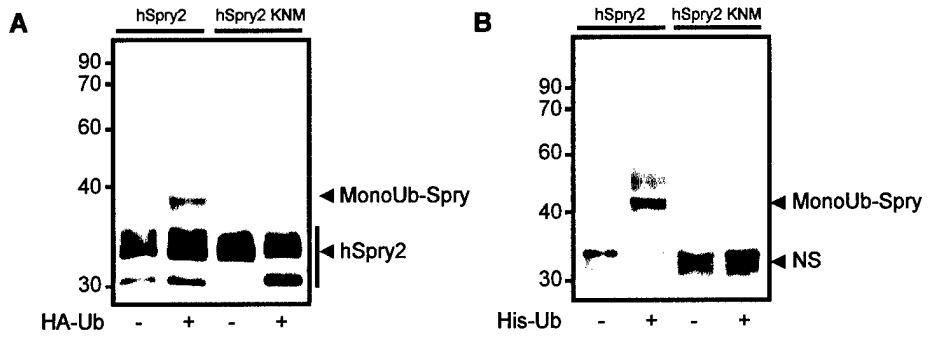

Figure 1. CHOK1 cells were transiently transfected with expression vectors encoding the HA-tagged hSpry2 FL or hSpry2 KNM, and either (A) HA-Ubiquitin (HA-Ub) or (B) Histidine-Ubiquitin (His-Ub). A total of 24 hr posttransfection, (A) the cells were lysed and analyzed by immunoprecipitation with anti-Spry antibody and Western blotting with anti-HA antibody. (B) Histidine-tagged proteins were purified using nickel-affinity chromatography and hSpry2 proteins detected by immunoblotting with anti-HA antibody. NS: Non-specific band.

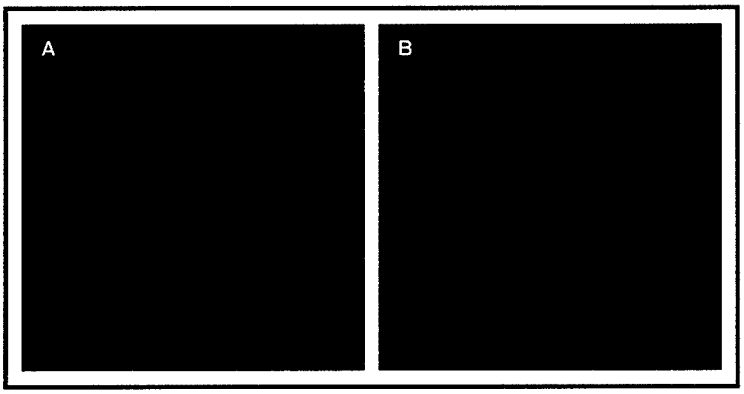

Figure 2. BHK cells were transiently transfected with either hSpry2 (A) or hSpry2 KNM (B). Cells were fixed in 3.7% formaldehyde in PBS for 1hr and were then permeabilized for 3 min at room temperature with PBS containing 0.1% Triton X-100. Staining with primary antibody (anti-HA) were carried out for 1hr at 37°C in PBS containing 1% BSA. Cells were rinsed with PBS and staining with secondary antibody (rhodamine-conjugated goat anti-mouse) were carried out for 1 hr at 37°C in PBS containing 1% BSA. Blue: DAPI staining to show nucleus.

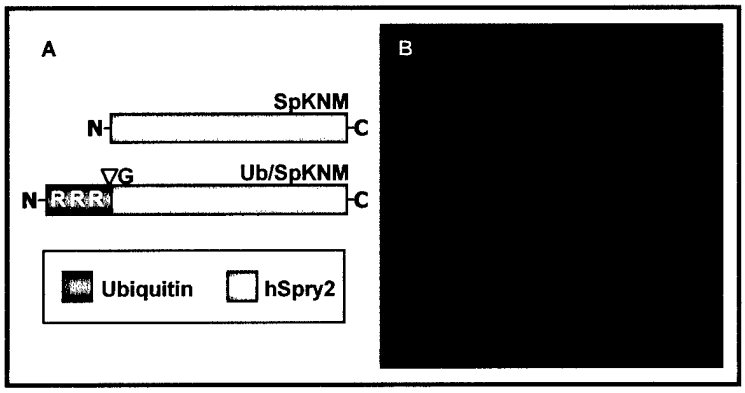

Figure 3. (A) Schematic representation of Ub/SpKNM construct. R and G represent Arginine and Glysine residue, respectively. (B) BHK cells were transiently transfected with Ub/SpKNM construct. Immunostaining was carried out as explained above.

Key Research Accomplishments

1. Generated an hSpry2 ubiquitination deficient mutant (hSpry2 KNM).
2. Performed an immunofluorescent microscopy to characterize the mutant phenotype.
3. Rescued the mutant phenotype by fusing a single ubiquitin moiety to hSpry2 KNM.

Reportable Outcomes

Hong Joo Kim and Dafna Bar-Sagi (2003), Modulation of signaling by Sprouty: A developing Story, Nat Rev Mol Cell Biol **5**, 441-450.

Conclusion

The sorting of proteins to distinct cellular compartments is required for many cellular processes. The primary function and the activity of cellular proteins are limited by their cellular destination. Therefore understanding the mechanisms that control protein sorting should provide insights into their biological function. Work performed over the past year has clearly demonstrated that the intracellular trafficking of hSpry2 is regulated by mono-ubiquitination. Further investigation into the functional importance of hSpry trafficking will lead to advance our understanding of the modulation of growth factor signaling.

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3/98-2/99	Cancer Research Center, Seoul National University, Research Assistant in the laboratory of Dr. Jeong-Sun Seo Characterization of molecular structure and function of mouse *clk-1* gene
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Publication

Hong Joo Kim and Dafna Bar-Sagi (2003), Modulation of signaling by Sprouty: A developing Story, Nat Rev Mol Cell Biol **5**, 441-450.

Presentation

Poster presentation	19th Annual Meeting on Oncogene, 2003 "Regulation of hSpry2 function by RTK signaling"
Oral presentation	PPG Meeting, SUNY at Stony Brook, 2002 "The dimerization of human *sprouty 2*"

MODULATION OF SIGNALLING BY SPROUTY: A DEVELOPING STORY

Hong Joo Kim and Dafna Bar-Sagi‡*

Sprouty proteins are evolutionarily conserved inducible inhibitors of signalling by receptor tyrosine kinases. They have been implicated in negative-feedback interactions that impart spatial and temporal constraints to intracellular signals. The repressive function of Sprouty proteins targets several receptor-tyrosine-kinase-dependent signalling steps, indicating that their activities might have broader biological consequences than was initially anticipated.

SH2 DOMAIN
(Src-homology-2 domain). A protein motif that recognizes and binds tyrosine-phosphorylated sequences, and thereby has a key role in relaying cascades of signal transduction.

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Receptor tyrosine kinases (RTKs) are key mediators of signal transmission in response to extracellular cues that regulate cell differentiation, proliferation and survival. RTK-mediated signalling events must be precisely regulated spatially and temporally to ensure a physiologically appropriate biological outcome. Indeed, the dysregulation of RTK signalling is causally linked to a large number of disease states. Recent studies have underscored the importance of feedback control of RTK function as a mechanism for ensuring signalling patterns that are compatible with the induction of a particular cellular response. Many of the insights into feedback regulation of RTK signalling have been attained from genetic analyses of developmental processes[1]. Instructive signals that control cell-fate specification during animal development are frequently modulated through the establishment of negative-feedback loops. A salient feature of these loops is the transcriptional induction of negative regulators by the very pathways that are eventually inhibited, which thereby provides an effective mechanism for the coordination of signalling input with the physiological response. One such negative regulator is Sprouty (SPRY), a recently identified repressor of RTK signalling in vertebrates and invertebrates. Although at first glance it seemed that the antagonistic function of SPRY could be easily explained, it is becoming increasingly evident that the mode of action of SPRY is multifaceted and subject to complex regulation. In this review, we will focus on the current understanding of the role of SPRY in modulating signalling events downstream of RTKs, and on the directions that research on SPRY proteins is likely to take in the future.

Sprouty structure

Drosophila melanogaster Spry (*D.m.* Spry) — the first member of the SPRY family of proteins to be discovered — is a 63-kDa protein that contains a unique 124-residue cysteine-rich region (FIG. 1). Outside the cysteine-rich region, *D.m.* Spry contains many stretches of repeating or alternating amino-acid residues, as well as a predicted sequence peptide near its amino terminus[2]. Homologues of *D.m.* Spry, which have sequence similarity that is mostly limited to the cysteine-rich domain, have been identified in the African clawed frog (*Xenopus laevis*), chickens, mice and humans[2–6]. The mouse and human genomes each contain four *SPRY* genes (*SPRY 1–4*) encoding proteins that are considerably smaller in size (32–34 kDa) than *D.m.* Spry, and that show sequence divergence at their amino termini. Although the conserved cysteine-rich domain is present at the carboxyl terminus of all mammalian SPRY proteins (FIG. 1), outside the cysteine-rich region there is only one short region of sequence similarity between *D.m.* Spry and individual mammalian SPRY proteins. This region contains a conserved tyrosine residue, which mediates the interaction of SPRY with signalling molecules that contain Src-homology-2 (SH2) domains (see below). The sequence divergence between the amino termini of the four mammalian SPRY proteins might dictate their differential functions, potentially by mediating distinct protein–protein interactions. A cysteine-rich domain that shares sequence similarity with that of SPRY proteins has been recently identified in a family of negative regulators of RAS signalling called SPRY-related proteins with an EVH1

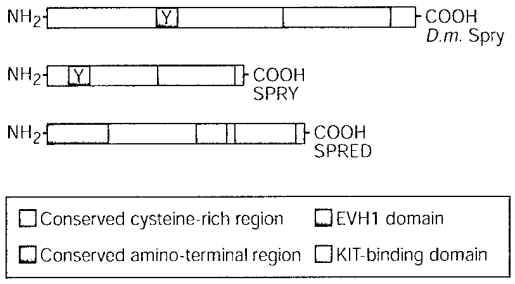


Figure 1 | **Sprouty and SPRED proteins.** The domain structures of *Drosophila melanogaster* Sprouty (*D.m.* Spry), mammalian Sprouty (SPRY) and mammalian SPRY-related protein with an EVH1 domain (SPRED). Y indicates the conserved tyrosine residue that undergoes phosphorylation in response to receptor-tyrosine-kinase stimulation. The carboxyl terminus of *D.m.* Spry and mammalian forms of SPRY, and SPRED, all contain a conserved cysteine-rich domain. The amino terminus of mammalian SPRY and *D.m.* Spry proteins only has one short region of sequence similarity, which contains a conserved tyrosine residue.

domain (SPRED proteins)[7] (FIG. 1). The functional relationships between SPRY and SPRED proteins will be discussed later in this article.

Sprouty expression

A common mechanism that is used to set up negative-feedback loops is the inducible expression of signalling pathway inhibitors through the same signalling pathways that they end up controlling. The expression patterns of SPRY during embryonic development are remarkably coincident with known sites of RTK signalling. An overt example of this coincidence is provided by the profile of SPRY expression in the developing lung. The embryonic lung undergoes a process known as branching morphogenesis, during which the tracheal epithelium is remodelled in response to inductive signals that originate in the mesenchymal compartment of the lung[8]. One such class of inductive signal involves fibroblast growth factors (FGFs), a family of growth factors that signal through tyrosine kinase FGF receptors to activate a variety of intracellular pathways that regulate pattern formation. FGFs have an essential role in the spatial coordination of the regulatory interactions between the epithelium and the mesenchyme in the embryonic lung by virtue of being expressed at discrete mesenchymal sites that are juxtaposed to the developing epithelium[9,10]. The expression of SPRY2 and *D.m.* Spry is induced within a subpopulation of epithelial cells at a developmental stage when the cells are highly responsive to FGF, which indicates that SPRY expression might be dependent on FGF signalling in both *D. melanogaster* and mammals[2,11–13]. The finding that Spry expression in the developing lung of the mouse is abolished when FGF signalling is compromised[2,11] lends further credence to this idea.

A close spatial and temporal interdependence between FGF signalling activity and *SPRY* gene expression has also been seen in various other mammalian embryonic tissues, including brain, heart, gut and muscle[3,14]. Similarly, *D.m. Spry* expression is induced by epidermal growth factor (EGF) signalling during embryonic development, most notably in the eye disc, the wing IMAGINAL DISC and the follicle cells of the ovary[15–17].

The regulation of SPRY expression in adult tissue has not been extensively characterized. In mammalian fibroblasts, EGF or FGF stimulation induces the expression of SPRY2 and SPRY4 through the activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade (BOX 1), and in mammalian endothelial cells, the expression of SPRY1 and SPRY2 is induced by FGF stimulation[18–20]. Altogether, the widespread dependence of SPRY expression on RTK signalling implies that SPRY participates in the negative-feedback control of signalling. However, SPRY expression is not always coupled to RTK signalling, and there are significant differences between the expression domains of individual mammalian *SPRY* genes, especially up to mid-embryogenesis[3]. In addition, *Spry2* transcripts are present at high abundance in the brain, lung and heart of the adult mouse[21]. Therefore, although the transcriptional induction of *SPRY* can be viewed as an inhibitory response to RTK signalling, it seems that the decision of how, when and where to engage this response is dependent on several factors, many of which remain to be determined. A necessary step towards accomplishing this goal is the identification of the regulatory elements that control the transcription of *SPRY* genes. The first insight into the identity of such elements is provided by the identification of *SPRY1* as the direct target of the kidney-specific transcription factor Wilms'-tumour-suppressor-gene-1 (WT1)[22].

Sprouty localization

Under most circumstances, the subcellular localization of a protein provides clues to its mode of action and/or function. It has been difficult to apply this concept to SPRY because of the numerous cellular sites at which this protein has been reported to reside. In *D. melanogaster*, ectopically expressed *D.m.* Spry localizes primarily to the plasma membrane and to EGF-receptor-containing structures in the cytoplasm[15]. A similar localization pattern has been shown for ectopically expressed SPRY2 in mammalian cells[23,24]. The cytoplasmic structures with which SPRY2 is associated are thought to represent an endosomal compartment, because they become enriched with endocytosed EGF-receptor molecules after exposure to EGF[24]. In a different set of studies, SPRY2 has been shown to colocalize with microtubules and to translocate to MEMBRANE RUFFLES in response to growth-factor stimulation[25–27]. The reasons for the discrepancies in SPRY2 localization patterns are not understood, and might reflect a biased targeting to a given cellular site as a result of differences between the levels of ectopic expression of SPRY2 that were attained in the different studies.

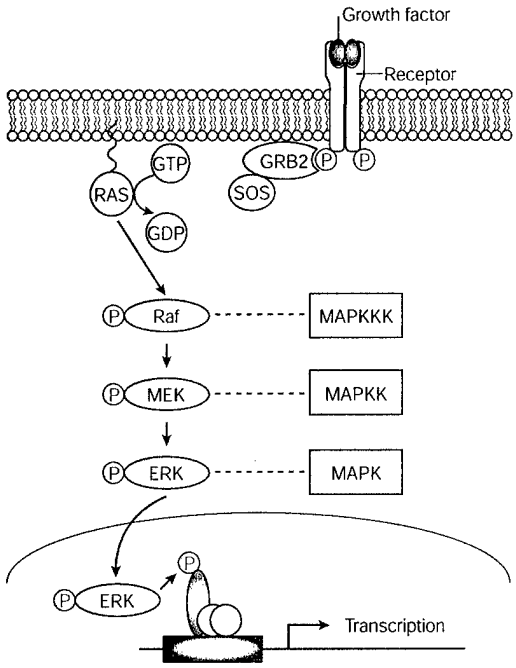
In unstimulated human umbilical vein endothelial cells (HUVECs), endogenous SPRY1 is found predominantly in perinuclear regions and in cytoplasmic vesicular structures[18]. After growth-factor stimulation, a small

IMAGINAL DISC
A single-cell layer epithelial structure of the *D. melanogaster* larva that gives rise to wings, legs and other appendages.

MEMBRANE RUFFLE
A process that is formed by the movement of lamellipodia that are in the dynamic process of folding back onto the cell body from which they previously extended.

Box 1 | **The ERK/MAPK cascade**

Mitogen-activated protein kinase (MAPK) cascades are ubiquitous signalling modules that couple receptor-mediated events at the cell surface to cytoplasmic and nuclear effectors. All MAPK cascades use a common signal-relay mechanism that involves the sequential phosphorylation of three kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK[61]. The first MAPK cascade to be characterized in vertebrates was the ERK/MAPK cascade (see figure). The acronym ERK stands for extracellular signal-regulated kinase and is derived from the fact that a variety of extracellular signals activate this cascade. The ERK/MAPK cascade is perhaps best known for its crucial role in mediating the transduction of signals from receptor tyrosine kinases (RTKs). The engagement of the ERK/MAPK cascade in response to ligand binding to RTKs is initiated by the activation of the small GTPase RAS. This step is accomplished by the recruitment of a protein complex consisting of the RAS exchange factor son-of-sevenless (SOS) and the growth-factor-receptor bound protein-2 (GRB2) to a tyrosine phosphate docking site on the receptors themselves or on receptor-substrate proteins[62]. Activated RAS triggers the activation of the MAPKKK RAF. Activated RAF then phosphorylates the MAPKK MEK (MAPK and ERK kinase) on serine residues leading to its activation. Subsequently, activated MEK catalyses the dual phosphorylation of the MAPK ERK. Phosphorylated ERK translocates to the nucleus where it phosphorylates and activates transcription factors that control the expression of genes that are required for cell growth, differentiation and survival.



LAMELLIPODIA
A thin, sheet-like cell extension that is found at the leading edge of crawling cells or growth cones.

LEADING EDGE
The thin margin of a lamellipodium that spans the area of the cell from the plasma membrane to about 1 μm back into the lamellipodium.

CAVEOLAE
Specialized rafts that contain the protein caveolin and form flask-shaped, cholesterol-rich invaginations of the plasma membrane that might mediate the uptake of some extracellular materials and are probably involved in cell signalling.

PLECKSTRIN HOMOLOGY (PH) DOMAIN
A sequence of 100 amino acids that is present in many signalling molecules and binds to lipid products of phosphatidylinositol 3-kinase.

PALMITOYLATION
The covalent attachment of a palmitate (16-carbon, saturated fatty acid) to a cysteine residue through a thioester bond.

EPISTASIS
A description of the relationship between alleles of different genes. Epistasis analysis is often used by geneticists to order genes in a pathway. When an animal that is a double mutant for two different genes displays the phenotype of one of the single mutants and not the other, the gene which is responsible for the observed phenotype is said to be epistatic.

fraction of SPRY1 translocates to the plasma membrane, mainly to the LAMELLIPODIA at the LEADING EDGE of the cells. The localization of endogenous SPRY1 shows a partial overlap with that of caveolin-1, a main constituent of CAVEOLAE, particularly in the perinuclear region and plasma membrane of HUVECs[18], but the significance of this spatial coincidence is unknown.

The carboxyl terminus and membrane targeting. Irrespective of their final destination, SPRY proteins are dependent on the conserved carboxy-terminal cysteine-rich domain for their localization[15,26–28]. Furthermore, this domain is sufficient for the targeting of *D.m.* Spry and SPRY2 to the plasma membrane[15,26]. The membrane-targeting domain of human SPRY2 has been defined as the minimal region that is necessary and sufficient for plasma-membrane localization. It encompasses residues 178–221. It binds to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) *in vitro* and colocalizes with the PLECKSTRIN HOMOLOGY (PH) DOMAIN of phospholipase C-δ (PLCδ)[26]. Moreover, a reduction in the cellular levels of PtdIns(4,5)P_2 — by various means — prevents the membrane localization of human SPRY2, which indicates that the cysteine-rich domain of SPRY directs membrane association probably by mediating PtdIns(4,5)P_2 binding[26]. Ectopically expressed SPRY1 and SPRY2 undergo PALMITOYLATION in HUVECs[18]. This modification could also be important in the membrane-anchoring mechanism of SPRY.

Membrane association is required for Sprouty function. Mutations that disrupt SPRY localization interfere with its function. For example, truncation of the cysteine-rich domain of *D.m.* Spry causes lethality[2]. In addition, a point mutant of human SPRY2 that contains an arginine-to-aspartic-acid substitution at position 252 is defective in membrane interaction and is impaired in its ability to inhibit FGF-induced ERK/MAPK activation[26]. Likewise, deletion mutants of SPRY2 that lack the membrane-targeting domain fail to negatively modulate cell migration, ERK/MAPK activation and cell proliferation in response to different growth factors[27,28]. The functional requirement for the membrane association of SPRY might reflect the fact that several SPRY targets (for example, GRB2 (growth-factor-receptor bound-2) and RAF) reside in the plasma membrane (FIG. 2).

Sprouty and antagonism of RTK signalling

Site of action. Genetic experiments using gain- or loss-of-function mutants of components of the signalling pathway from RTKs to ERK/MAPK have indicated that the inhibitory activity of SPRY is exerted upstream of ERK/MAPK and downstream of the RTK. However, it seems that the precise point at which SPRY intercepts RTK signalling can vary depending on the biological context (FIG. 3). In the developing *D. melanogaster* eye, EPISTASIS studies place *D.m.* Spry upstream of Ras and downstream of the EGF receptor[2], whereas in the developing wing and ovary, *D.m.* Spry functions at the level of Raf, or downstream of it[17]. In mouse fibroblasts, Spry1 and Spry2 interfere with growth-factor-induced ERK/MAPK

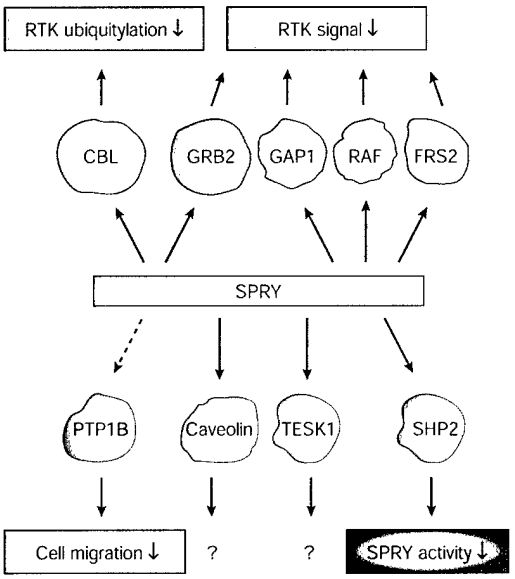


Figure 2 | **Sprouty interactions.** Proteins that interact with SPRY physically (solid arrows) or functionally (broken arrow). Where known, the functional consequences of the interactions are indicated in the boxed areas. Note that the position of the different interacting proteins relative to the SPRY molecules does not signify the binding site on SPRY. FRS2, fibroblast-growth-factor-receptor substrate-2; GRB2, growth-factor-receptor bound-2; PTP1B, protein-tyrosine phosphatase-1B; RTK, receptor tyrosine kinase; SHP2, Src-homology-2-domain-containing protein tyrosine phosphatase-2; TESK1, testis-specific protein kinase-1.

signalling at the level of Ras activation[29], whereas in human epithelial cells, SPRY2 functions at the level of RAF activation[30]. Likewise, mammalian SPRY4 functions at the level of RAF to inhibit ERK/MAPK activation that is induced by vascular endothelial growth factor (VEGF)[31].

Of all the SPRY proteins studied to date, *X. laevis* Spry2 represents an exception, in that its expression leads to the impairment of FGF-dependent pathways that control gastrulation movements, but it has no effect on ERK/MAPK activity[6]. Significantly, *D.m.* Spry shows the same effect when expressed in *X. laevis*, which further supports the idea that SPRY has the capacity to target RTK signalling at many levels and that the specific placement of SPRY in the pathway is dictated by the cellular context.

Potential modes of action. Our understanding of the molecular basis for the negative modulation of RTK signalling by SPRY is still at a preliminary stage. The experimental evidence that has been gathered so far points to several mechanisms, all of which involve the interaction of SPRY with essential elements of the RTK–RAS–ERK/MAPK cascade (FIG. 3). In their amino-terminal domain, SPRY proteins contain a conserved tyrosine residue (FIG. 1; Tyr55 in human SPRY2) that undergoes phosphorylation in response to growth-factor stimulation[23,24,28,32]. This phosphorylation site functions as a binding site for the SH2 domain of the adaptor molecule GRB2 (REF. 28). In the context of FGF signalling, the FGF-induced interaction of SPRY with GRB2 has two consequences. It prevents GRB2 from binding to either FGF-receptor substrate-2 (FRS2) or SH2-domain-containing protein tyrosine phosphatase-2 (SHP2)[28]. The SH2-dependent recruitment of complexes of GRB2 and SOS (son-of-sevenless) to FRS2 and SHP2 is a necessary step in the coupling of FGF-receptor stimulation to RAS activation. So, the sequestration of GRB2 by SPRY would be predicted to inhibit signalling events downstream of RAS (FIG. 3). This prediction is supported by the observation that a phosphopeptide corresponding to the tyrosine phosphorylation site of SPRY2 competes effectively with FRS2 and SHP2 for binding to GRB2 *in vitro* and inhibits ERK/MAPK activation by FGF *in vivo*[28]. The identity of the kinase that is responsible for the tyrosine phosphorylation of SPRY in response to FGF stimulation is not known.

The carboxy-terminal cysteine-rich domain of SPRY proteins contains a conserved region of approximately 70 residues, which interacts with the catalytic domain of RAF *in vivo* and *in vitro*[31]. This interaction blocks the activation of RAF1 by protein kinase C-δ (PKCδ), but has no effect on the RAS-dependent activation of RAF1. The significance of this interaction has been evaluated by examining the effects of SPRY4 on VEGF-induced ERK/MAPK activation, a process that is mediated by the PLCγ1–PKC pathway and is independent of RAS[31]. The ectopic expression of SPRY4 inhibits the PKC-dependent activation of ERK/MAPK in response to VEGF[31]. By contrast, a deletion mutant of SPRY4 that lacks the RAF-interacting region is unable to suppress VEGF-induced ERK/MAPK activation, which indicates that the interaction between SPRY4 and RAF1 is necessary for the inhibitory activity of SPRY4 (FIG. 3).

D.m. Spry binds to a negative regulator of Ras, Gap1, *in vitro* and the resulting eye phenotypes of *D.m. Spry* and *Gap1* mutants are very similar. On the basis of these observations, it has been proposed that *D.m.* Spry recruits Gap1 to Ras-containing signalling complexes, thereby accelerating the inactivation of Ras[15]. In mammalian cells, SPRY2 associates with GAP1 and this association is substantially decreased after FGF stimulation[33]. It remains to be determined whether the reversible interaction of SPRY with GAP1 contributes directly to the negative regulation of ERK/MAPK activation.

Although the interactions of SPRY with GRB2, RAF and, to a lesser extent, with GAP1 provide a mechanistic framework for understanding how SPRY expression can lead to the suppression of RTK signalling at the level of RAS or RAF, other functional attributes of SPRY cannot be explained solely on the basis of these interactions. For example, in the *D. melanogaster* tracheal system, *D.m.* Spry functions non-cell-autonomously to repress FGF signalling in cells that are adjacent to the cells in which *D.m.* Spry is expressed[2] (see later). Although *D.m.* Spry can bind to Downstream of receptor kinases (Drk), the *D. melanogaster* homologue of GRB2 (REF. 15), it is difficult to envisage how this interaction — which, by its very nature, is intracellular — could account for a non-cell-autonomous effect. Additionally, in certain

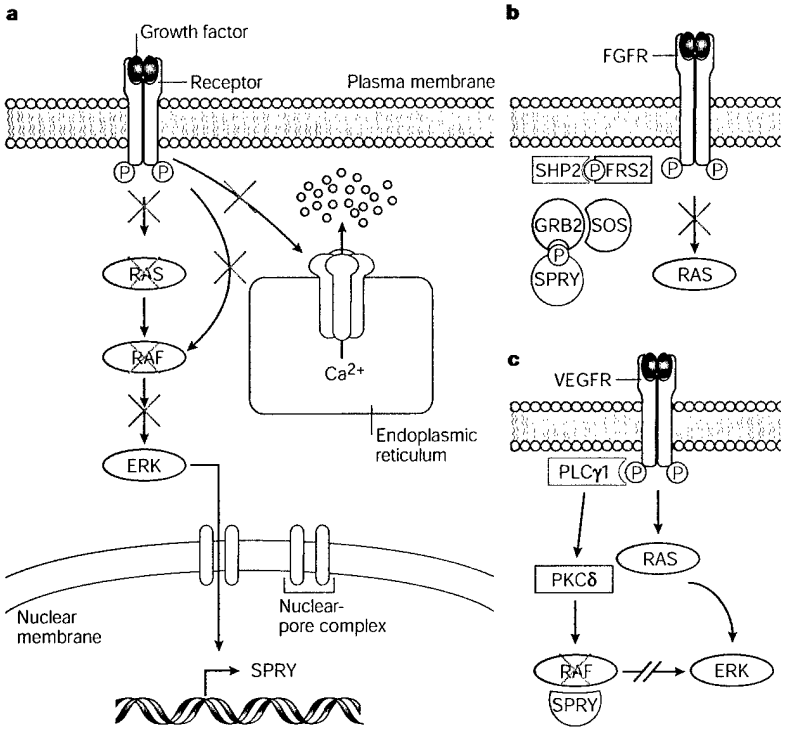


Figure 3 | **Repression of RTK signalling by Sprouty. a** | Schematic presentation of two effector pathways that are activated downstream of receptor tyrosine kinases (RTKs) and that are antagonized by Sprouty (SPRY): the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade (left) and intracellular calcium mobilization (right). The activation of the ERK/MAPK cascade induces the transcriptional upregulation of Sprouty (SPRY). Depending on the signalling context (see text), SPRY can interfere with RTK pathways at many levels, as indicated (red crosses). **b** | Activation of the fibroblast growth factor (FGF) receptor (FGFR) induces the tyrosine phosphorylation of FGFR substrate-2 (FRS2) or Src-homology-2 (SH2)-domain-containing protein tyrosine phosphatase-2 (SHP2). In the absence of SPRY, a complex of growth-factor-receptor bound-2 (GRB2) and son-of-sevenless (SOS) is recruited to the phsphorylated tyrosine residues on FRS2 or SHP2 through the SH2 domain of GRB2. This recruitment event results in the activation of RAS. FGF stimulation also leads to tyrosine phosphorylation of SPRY, thereby generating a binding site for the GRB2–SOS complex. Therefore, the presence of SPRY prevents the recruitment of the GRB2–SOS complex to FRS2 or SHP2 and, as a consequence, RAS activation is inhibited. **c** | ERK activation by vascular endothelial growth factor (VEGF) is mediated by phospholipase C-γ1 (PLCγ1)-induced activation of protein kinase C-δ (PKCδ), which, in turn, phosphorylates and activates RAF. SPRY binds, through its conserved cysteine-rich domain, to RAF and this interaction inhibits the phosphorylation and activation of RAF and subsequent activation of ERK. P, phosphate; VEGFR, VEGF receptor.

cells, SPRY can inhibit ERK/MAPK activation in the absence of tyrosine phosphorylation[18,28] or without affecting the recruitment of GRB2–SOS complexes to FRS2 (REF. 29), which indicates that there might be alternative mechanisms that are used by SPRY to antagonize RTK signalling.

Sprouty and EGF signalling

In *D. melanogaster*, *D.m.* Spry functions as an inhibitor of EGF-receptor signalling, as indicated by the following observations: halving the dose of *D.m.* Spry enhances the phenotype that is induced by a constitutively active form of the EGF receptor; and overexpression of *D.m.* Spry rescues the phenotype that is caused by a constitutively active EGF receptor[15,17]. By marked contrast, expression of mammalian SPRY proteins not only fails to repress EGF signalling but, under some circumstances, even potentiates the activation of this pathway[18,23,24,31,34,35]. This indicates that the mammalian SPRY homologues have probably evolved a distinct function in the context of EGF signalling. Unexpectedly, the molecular event that initiates the agonistic effect of SPRY on EGF signalling in mammals is identical to that which sets in motion the antagonistic effect of SPRY on FGF signalling. Similar to the effect of FGF stimulation, SPRY2 undergoes phosphorylation on Tyr55 in response to EGF stimulation[23,24,28,32]. However, in the context of EGF-receptor activation, this phosphorylation event augments an interaction between SPRY2 and the E3 UBIQUITIN LIGASE CBL[23,24,32] (FIG. 4). Normally, CBL binds to the activated EGF receptor and promotes its ubiquitylation and degradation[36], but the binding of SPRY2 to CBL inhibits this process[24,34,35]. As a consequence, downregulation of activated EGF receptor is prevented, and this leads to a prolonged signalling activity.

Potential modes of action. The mechanism by which SPRY interferes with the function of CBL has not been fully elucidated. According to two reports, the tyrosine phosphorylated form of SPRY2 binds to the SH2 domain of CBL[24,32]. As this SH2 domain is required for CBL to interact with the activated form of the EGF receptor, the binding of SPRY2 to CBL displaces CBL from the EGF receptor, thereby impairing receptor ubiquitylation and degradation. It is noteworthy that the phosphotyrosine residue on SPRY2 that functions as a binding site for CBL, Tyr55, is the same residue that mediates the interaction of SPRY2 with the SH2 domain of GRB2 (REF. 28). The latter interaction, as mentioned above, has been implicated in the suppressive effect of SPRY on FGF-receptor signalling[28]. So the physiological consequence of SPRY expression probably reflects the balance between these two competitive binding events.

Another mechanism to explain how SPRY might prevent CBL-mediated ubiquitylation of the EGF receptor postulates that the site of interaction of SPRY with CBL is the catalytic RING FINGER, a conserved domain that directs the ubiquitylation of substrate proteins through the recruitment of E2 UBIQUITIN-CONJUGATING ENZYMES (FIG. 4)[36]. The binding of SPRY2 to the RING-finger domain of CBL competes with the binding of the E2 ubiquitin-conjugating enzyme UBCH7, thereby compromising the ubiquitin ligase activity of CBL[35,37]. In contrast to the interaction of SPRY with the SH2 domain of CBL, the interaction of SPRY with the RING finger of CBL is specific, but is growth-factor independent[37]. So the binding of SPRY to the RING-finger and SH2 domains of CBL might account for the constitutive and regulated SPRY–CBL interactions, respectively (FIG. 4).

Sprouty degradation. The formation of a complex between SPRY and CBL impacts not only on the duration of EGF-receptor signalling, but also on the lifespan of SPRY itself. The EGF-dependent binding of SPRY2 to CBL promotes the multiubiquitylation and

E3 UBIQUITIN LIGASE
The third enzyme in a series — the first two are designated E1 and E2 — that is responsible for ubiquitylation of target proteins. E3 enzymes provide platforms for binding E2 enzymes and specific substrates, thereby coordinating ubiquitylation of the selected substrates.

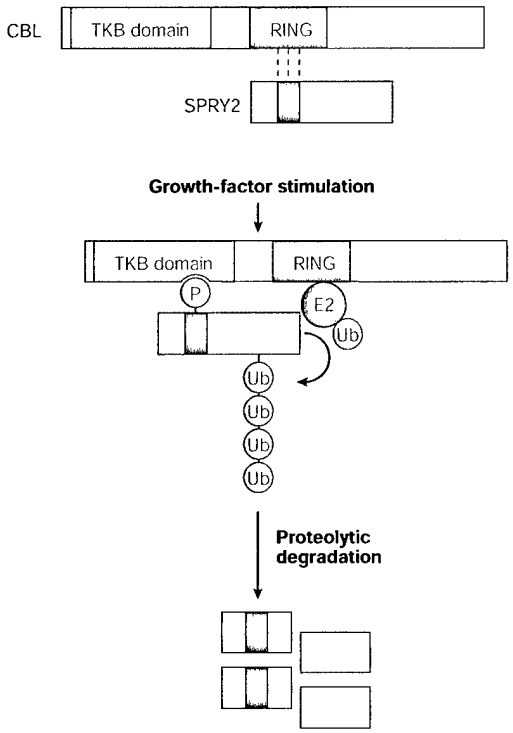


Figure 4 | **Sprouty–CBL interaction.** The main domains of CBL that are relevant for this interaction are the tyrosine kinase binding (TKB) domain and the RING-finger domain, which functions as an E3 ubiquitin ligase. In the absence of growth factors, SPRY2 binds weakly to the RING-finger domain of CBL through its conserved amino-terminal region (blue). After stimulation by growth factors, the interaction between SPRY2 and CBL becomes tighter and is mediated by the binding of a phosphorylated tyrosine residue on SPRY2 to the Src-homology-2 (SH2) domain in the TKB domain of CBL. This interaction is accompanied by the transfer of ubiquitin molecules (Ub) from the RING-finger-bound ubiquitin conjugating enzyme (E2) to SPRY2, which directs the proteolytic degradation of SPRY2.

RING FINGER
A protein domain that consists of two loops that are held together at their base by cysteine and histidine residues that form a complex with two zinc ions. Many RING fingers function in protein degradation by facilitating protein ubiquitylation.

E2 UBIQUITIN-CONJUGATING ENZYME
An enzyme that accepts ubiquitin from a ubiquitin-activating enzyme (E1) and, together with a ubiquitin ligase (E3), transfers it to a substrate protein.

PC12 CELLS
A clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor and can synthesize, store and secrete catecholamines, much like sympathetic neurons. PC12 cells contain small, clear synaptic-like vesicles and larger dense-core granules.

DOMINANT-NEGATIVE
A defective protein that retains interaction capabilities and so distorts or competes with normal proteins.

CHONDRODYSPLASIA
A general name for heterogenous disorders of skeletal development and growth that involves alterations in the architecture of epiphyseal growth and endochondral ossification.

proteolytic degradation of SPRY[23,24]. An accelerated degradation of SPRY has also been observed in response to FGF stimulation[23]. The downregulation of SPRY in response to RTK signalling might limit its repressive activity to a defined period following receptor activation. Such temporal restriction is crucial for many developmental processes in which cells need to respond repeatedly to the same ligand in order to become committed to a certain fate.

Biological consequences of Sprouty expression

Cell proliferation and differentiation. The extent and duration of growth-factor-induced ERK/MAPK activation are essential determinants in specifying the proliferative fate of cells. In fibroblasts, ERK/MAPK activity during mid-G1 phase is required for cell-cycle progression[38–40], but, in PC12 CELLS, prolonged ERK/MAPK activity is required to induce cell-cycle withdrawal and neuronal differentiation[41]. Because SPRY proteins have the capacity to repress ERK/MAPK activation by certain growth factors, it is perhaps not surprising that ectopic expression of SPRY results in the attenuation of fibroblast proliferation and PC12 differentiation[20,27,29]. The involvement of endogenous SPRY proteins in these processes has been tested using a DOMINANT-NEGATIVE form of SPRY in which the conserved amino-terminal tyrosine residue (Tyr55 in SPRY2, Tyr53 in SPRY4) is mutated[20,28]. This mutation compromises the ability of SPRY to interfere with ERK/MAPK activation because it abolishes GRB2 binding, but it has no effect on the subcellular localization of SPRY. The expression of this mutant in PC12 cells results in the potentiation of growth-factor-induced neuronal differentiation and ERK/MAPK activation. With respect to the latter, it is of interest to note that the potentiating effect is restricted to the prolonged phase of ERK/MAPK activation, which is consistent with Spry functioning as a transcriptionally induced negative-feedback regulator of the ERK/MAPK cascade. Significantly, the delayed phase of ERK/MAPK activation is the crucial phase for promoting neuronal differentiation[41].

Receptor trafficking. The endocytic sorting of activated RTKs is a highly regulated process that involves the ordered movement of receptor molecules through distinct vesicular compartments[42]. It is widely accepted that CBL-mediated ubiquitylation of RTKs has an important role in the targeting of activated RTKs to a specific endocytic pathway that culminates in their lysosomal degradation[43]. Therefore, a plausible outcome of the competition between SPRY and EGF receptors for CBL binding is the impairment of receptor trafficking[24,35]. Indeed, the expression of SPRY has been shown to inhibit the ligand-induced clearance of EGF receptors from the cell surface[24,35]. However, the specific endocytic steps that are directly influenced by the expression of SPRY need to be further defined. In addition, as CBL promotes the ubiquitylation and downregulation of several other growth-factor receptors, such as receptors for platelet-derived growth factor (PDGF), colony-stimulating factor-1 (CSF1) and FGF[44–46], it would be important to establish whether the endocytic sorting of these receptors is also affected by SPRY expression.

Bone development. Excess Spry expression in the chick limb leads to a decrease in size and thickening of skeletal elements[3]. This phenotype arises from a severe CHONDRODYSPLASIA in which the differentiation of chondrocytes is inhibited. The overall features of the chondrodysplasias induced by Spry expression are similar to those displayed in human chondrodysplasias that are caused by mutations in FGF-receptor-3 that render it constitutively active[47]. So in the context of bone development, the effects of SPRY overexpression mimic excess FGF signalling, which indicates that SPRY might have a potentiating, rather than a repressing, effect in this setting. Alternatively, SPRY expression might antagonize other signalling pathways that are required for bone formation. The identity of these pathways remains to be determined.

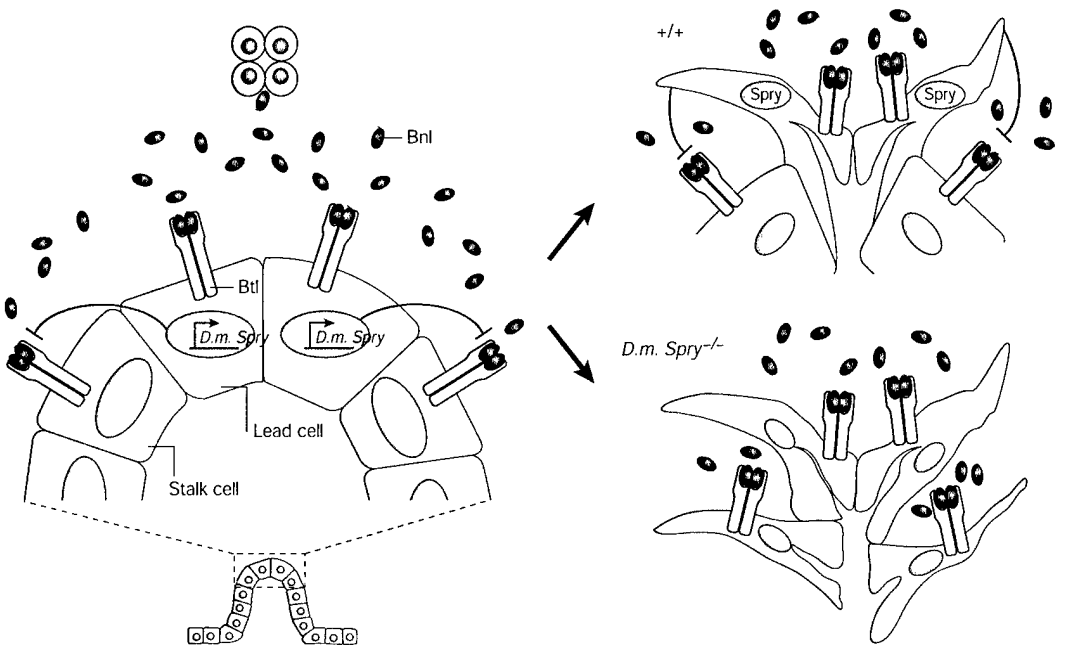


Figure 5 | **The role of Sprouty in branching morphogenesis during *Drosophila melanogaster* tracheal development.** Branchless (Bnl), a *Drosophila melanogaster* fibroblast growth factor (FGF) that is secreted from discrete sets of cells surrounding the tracheal epithelium (red), binds and activates the breathless (Btl) FGF receptor on the tracheal cells. The activation of Btl initiates the remodelling of the epithelium to produce primary branches consisting of lead cells (pink) followed by stalk cells (grey). Bnl also induces the expression of *D. melanogaster Sprouty* (*D.m. Spry*) selectively in the lead cells. Several hours after the budding of primary branches, secondary branches begin to form. In wild-type embryos ($^{+/+}$) *D.m.* Spry functions in a non-cell-autonomous way to inhibit Bnl signalling in the stalk cells, thereby restricting secondary-branch formation to the lead cells. In *D.m.* Spry loss-of-function mutants (*D.m. Spry*$^{-/-}$), this spatial restriction is lost and, as a result, the tracheal stalk cells adopt a branching fate.

Calcium mobilization. The diverse functions of RTKs are mediated through the activation of several biochemical pathways, one of which involves an increase in the levels of cytoplasmic calcium as a result of the opening of intracellular calcium channels[48]. This process is triggered by the PLC-mediated cleavage of PtdIns(4,5)P_2, which yields inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3). This then binds and activates the Ins(1,4,5)P_3 receptor/calcium channel. In *X. laevis* oocytes, activation of the FGF receptor leads to the rapid mobilization of intracellular calcium and this effect is blocked by the expression of *X. laevis* Spry[6]. By contrast, *D.m.* Spry has no effect on calcium mobilization when expressed in *X. laevis* oocytes[6]. Presumably, this functional difference reflects the low degree of sequence conservation between the two proteins. However, *X. laevis* Spry is highly related to mammalian SPRY proteins, which raises the testable hypothesis that some of the effects of SPRY in mammalian cells might be linked to suppression of calcium mobilization.

Branching morphogenesis. The term 'branching morphogenesis' refers to the formation of tree-like networks of epithelial tubes through reiterated cycles of branch initiation, branch outgrowth and branch arrest. This process relies on the precise spatio–temporal control of gene expression, cell proliferation and migration, and is essential for the physiological function of many organs including the lung, the vascular system and the kidney[49]. Many of the key insights into the mechanisms that specify branching pattern have been derived from the genetic dissection of *D. melanogaster* tracheal development. Branching of the respiratory trachea in *D. melanogaster* is initiated from epithelial sacs through the budding of primary branches, which consist of lead cells followed by stalk cells (FIG. 5). The lead cells at the growing ends of the primary branches form secondary branches, which subsequently extend several cytoplasmic projections to form terminal branches[8]. Both primary and secondary branching are patterned by the secretion of the *D. melanogaster* FGF — branchless (Bnl) — from clusters of cells that are located at specific sites near the tracheal epithelium[9]. The secreted Bnl activates the breathless FGF receptor (Btl) on the surface of the tracheal cells and the signalling events that ensue induce the remodelling of the epithelium to produce branches[50–53]. *D.m.* Spry is induced by Bnl selectively in the lead cells at the tip of the primary branches, and it inhibits Bnl signalling in the nearby stalk cells, thereby restricting the sprouting of secondary branches to the apical end of the primary branches (FIG. 5). In accordance with this function, *D.m.* Spry mutations result in the excessive branching from stalk cells due to overactivity of the Bnl pathway[2].

The patterning programmes that direct *D. melanogaster* tracheal development and vertebrate lung development are highly similar, especially with respect to the essential role that FGF signalling has in regulating branching morphogenesis. Mouse Spry2 is expressed in the lung epithelium at discrete sites that coincide with areas of high FGF signalling activity[21]. Reducing mouse *Spry2* expression by the introduction of specific antisense oligonucleotides into lung organ cultures leads to a significant enhancement in branching morphogenesis[21], whereas targeted overexpression of *Spry* in the peripheral lung epithelium impedes branching[11,54]. SPRY is therefore an essential component of a highly conserved regulatory circuitry that controls branching patterns through the spatial restriction of FGF signalling.

Similar to the process of tracheal and lung branching, the formation of new blood vessels (angiogenesis) during the development of the cardiovascular system also involves the sequential rounds of branching of endothelial cells[55]. Signalling through the VEGF and FGF RTKs is a well-established component of angiogenic regulation[56]. The expression of mouse Spry proteins in cultured endothelial cells inhibits FGF- and VEGF-induced proliferation, differentiation and migration by repressing signalling pathways that lead to ERK/MAPK activation[18,57]. Moreover, overexpression of Spry in the vasculature of mouse embryos inhibits blood-vessel branching during *in vivo* angiogenesis[57]. These observations indicate that the expression of SPRY in endothelial cells is likely to have a profound effect on the angiogenic process. In this context, it is of interest to note that SPRY was found to be upregulated during capillary morphogenesis in three-dimensional collagen matrices[58]. Collectively, the implication of SPRY in the regulation of branching morphogenesis adds a new twist to a well-established model for the generation of patterning information through negative-feedback control.

Cell motility. The involvement of SPRY in regulating the migratory behaviour of cells is indicated by two sets of observations. First, the migration-enhancing effect of several growth factors, including EGF, FGF and PDGF, is abrogated by the expression of human SPRY2 in HELA CELLS[27]. Second, *X. laevis* Spry2 inhibits the FGF-dependent polarized movement of mesodermal cells that is necessary for the formation and elongation of the embryonic body axis during *X. laevis* gastrulation[6]. In both cases, the conserved cysteine-rich domain is required for the inhibitory action of SPRY. A priori, the inhibitory activity of SPRY could rely on two mechanisms — a direct effect through the targeting of protein(s) that control cell movement, or an indirect effect resulting from the repression of RTK signalling. In HeLa cells, the anti-migratory effect of SPRY is mediated by the upregulation of the activity of protein-tyrosine phosphatase-1B (PTP1B)[59]. This is accompanied by the reduction in the tyrosine phosphorylation of several cellular proteins including $p130^{CAS}$, an important component of FOCAL-ADHESION COMPLEXES[59]. The mechanisms by which *X. laevis* Spry interferes with the induction of mesoderm morphogenesis by FGF-receptor signalling are not known.

HELA CELLS
An established tissue-culture strain of human epidermoid carcinoma cells, containing 70–80 chromosomes per cell. These cells were originally derived from tissue taken from a patient named Henrietta Lacks in 1951.

FOCAL-ADHESION COMPLEX
A flat, elongated structure, about 2–5 μm in size, that is found primarily at the cell periphery and mediates adhesion to the substrate by connecting the actin cytoskeleton with the extracellular matrix.

Relatives of Sprouty

By conducting a yeast two-hybrid screen for proteins that bind to the kinase domains of KIT and FMS, Wakioka and colleagues have identified two related proteins, SPRED1 and SPRED2, that bear structural and functional similarities to SPRY[7]. The structural similarity lies in the carboxy-terminal cysteine-rich domain of the two families of protein (FIG. 1). In addition, SPRED proteins contain an amino-terminal ENA/Vasodilator-stimulated phosphoprotein (VASP) homology-1 (EVH1) domain and a central 50-amino-acid domain that mediates the binding to KIT (KIT-binding domain, KBD). SPRED proteins are targeted to the plasma membrane by their cysteine-rich domain and undergo tyrosine phosphorylation in response to stimulation by several growth factors including CSF1, PDGF and EGF. Similar to SPRY, SPRED inhibits growth-factor-mediated ERK/MAPK activation[7]. The capacity of SPRED to exert this inhibitory effect depends on both the cysteine-rich and EVH1 domains and is accomplished through the suppression of RAF activation[7]. SPRED and RAS form a complex in the cells as judged by co-immunoprecipitation and colocalization studies[7]. However, this interaction has no effect on growth-factor-induced RAS activation. Rather, SPRED expression prolongs the association of RAF with the membrane and augments the binding of RAF to RAS, which indicates that SPRED expression might suppress RAF activation by altering the dynamics of RAF distribution and, as a consequence, its accessibility to activators.

SPRED is evolutionarily conserved, as indicated by the presence of a SPRED homologue, AE33, in *D. melanogaster*[60]. AE33 has been identified as a target of transcription factors that regulate photoreceptor-cell development but its biochemical function in *D. melanogaster* is not known. In mammalian cells, the function of SPRED most closely resembles that of SPRY4 in that both proteins intersect with growth-factor-induced ERK/MAPK activation at the level of RAF[7,31]. However, the biochemical mechanisms by which SPRY and SPRED inhibit RAF activity and the signalling context in which they operate are different. For example, SPRED inhibits ERK/MAPK activation by EGF, whereas SPRY4 has no antagonistic effect on EGF signalling[31]. Therefore SPRED and SPRY might provide complementary modalities for the modulation of RTK signalling.

Conclusions and future perspectives

In the short period that has elapsed since the discovery of SPRY proteins in 1998, significant progress has been made towards characterizing their involvement in the regulation of various RTK-dependent cellular processes. As discussed in this article, we now know that SPRY proteins can influence the intensity and duration of RTK signalling by intersecting with the ERK/MAPK cascade at several levels. Furthermore, the perturbation of RTK signalling that results from the expression of SPRY proteins produces a broad spectrum of effects that range from alterations in developmental fate to changes in cellular homeostasis. However, much

remains to be learnt with respect to the molecular mechanisms by which SPRY proteins function and the physiological consequences of their activity. Most of the information gathered so far regarding the function of SPRY in vertebrates has been derived from overexpression studies. The use of gene-targeting methods, at the cellular or organismal levels, is expected to provide crucial insights into the mechanistic basis of the interactions of SPRY proteins with elements of the signal-transduction machinery.

It is becoming readily apparent that the function of SPRY proteins has a built-in complexity that results from the large number of SPRY isoforms and binding partners. How SPRY-mediated protein–protein interactions are specified and subsequently used to exert effects on the potency of RTK signalling remains to be established. A prominent challenge in these efforts will be to identify determinants that confer context-dependent deployment of the activities of SPRY proteins. The integration of the different factors that contribute to the regulation of SPRY function into a testable, quantitative model of feedback regulation of RTK signalling should prove useful in untangling some of these complexities. Lastly, perturbations in RTK pathways are causally linked to a variety of pathological conditions. Therefore, the analysis of the role of SPRY in regulating the signalling output of RTKs under pathological conditions will enhance our understanding of the molecular links between the dysregulation of RTK signalling and human disease.

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Competing interests statement

The authors declare that they have no competing financial interests.

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